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**Altered Astrocyte-Neuron Interactions and Epileptogenesis in Tuberous Sclerosis Complex Disorder**

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<p><b>14. ABSTRACT</b> While the molecular basis of TSC is well established, far less is known about the pathogenetic mechanisms of epileptogenesis in TSC, which is found in the vast majority of patients. We introduce the novel concept that this follows from a disruption of normal synaptic pruning that is due to TSC mutation in astrocytes, the major non-neuronal cell type of the brain. Previous studies mostly focused on focal epileptogenesis in cortical tubers. In this study, we propose that <i>nontuber cortex may present an abnormally excitable neuronal network that could underlie seizure generation</i>. We will concentrate on a non-neuronal mechanism may exist in regulating synaptic function and thus epileptogenesis in TSC. To explore this aim, we will use a wide variety of experimental approaches, including electrophysiological (patch-clamping in culture cells and slice preparations to in vivo video-EEG monitoring), histological (conventional and fluorescent assays), molecular biological (Western blotting, biotinylation), and modern cellular imaging (confocal and two-photon microscopy of tissue preparations) techniques. We will measure biomarkers for astrocyte and neuronal functions and provide an index of glial transmission in the cortex of TSC deficient mouse models and peri-tuber or non-tuber tissue from TSC human brain. We will determine whether astrocyte dysfunction will lead to a failure in pruning excessive excitatory synapses during development, which underlies epilepsy in young TSC patients.</p>					
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# **Altered Astrocyte-Neuron Interactions and Epileptogenesis in Tuberous Sclerosis Complex Disorder**

## **Introduction**

This report is on the first year of our 2011 Tuberous Sclerosis Complex Research Program Idea Development Award (07/12-6/15). The major goals are to explore the potential mechanism for epileptogenesis in Tuberous Sclerosis Complex (TSC) disease, with a focus on altered astrocyte-neuronal interactions caused by astrocyte specific TSC deficiency.

The major findings in our animal models are that a) TSC1 mGFAPCre CKO mice develop spontaneous seizures; b) TSC1CKO mice show astrogliosis, activated mTOR signaling, enlarged cells size for neurons and astrocytes; C) glutamate transport and potassium buffering are intact in TSC1 mGFAPCre CKO astrocytes; D) TSC1CKO mice exhibit abnormal excitatory synaptic transmission; E) synaptic damage is induced by spontaneous seizures F) there is an increased spine density before the occurrence of spontaneous seizures.

As glutamate and potassium uptake remains intact in TSC1CKO astrocytes in our new mouse model, alternative mechanisms may explain how TSC1CKO astrocytes contribute to epileptogenesis. These include a deficiency of GABAergic interneurons and enhanced excitatory synaptic connectivity. We are now in the process of studies proposed in our second aim, "Analyze whether astrocyte dysfunction involves in postnatal spine pruning defect, which may results in abnormally enhanced excitatory synaptic connectivity and epileptogenicity", and the third aim "Analyze tuber, peri-tuber and non-tuber tissue from TS and control human brain tissue for mTOR, astrocyte function and synaptic formation".

## Body

### Background

TSC is a multisystem genetic disorder characterized by the presence of benign tumors (hamartomas) caused by mutations in one of the two genes, TSC1 and TSC2, which encode hamartin and tuberin respectively, and lead to hyperactivity of mammalian target of rapamycin (mTOR). The pathological hallmark of TSC brains are cortical tubers, characterized by disorganized cortical lamination, aberrant dendritic arbors and axonal projections, astrocytic proliferation, and abnormal cell morphology (ie, dysplastic and heterotopic neurons and giant cells). Epilepsy is the most common neurological manifestation in TSC, affecting up to 92% of people with TSC, which is refractory to treatment.

While the molecular basis of TSC is well established, far less is known about the pathogenetic mechanisms of epileptogenesis in TSC. Although seizures are associated with the number of cortical tubers, recent findings suggest that cortical tubers are not the epileptic foci in TSC brain, as 10% of people with TSC who have intractable tuberous sclerosis complex exhibit normal MRI findings. Furthermore, in approximately one third of individual with TSC, seizures persist even after surgery.

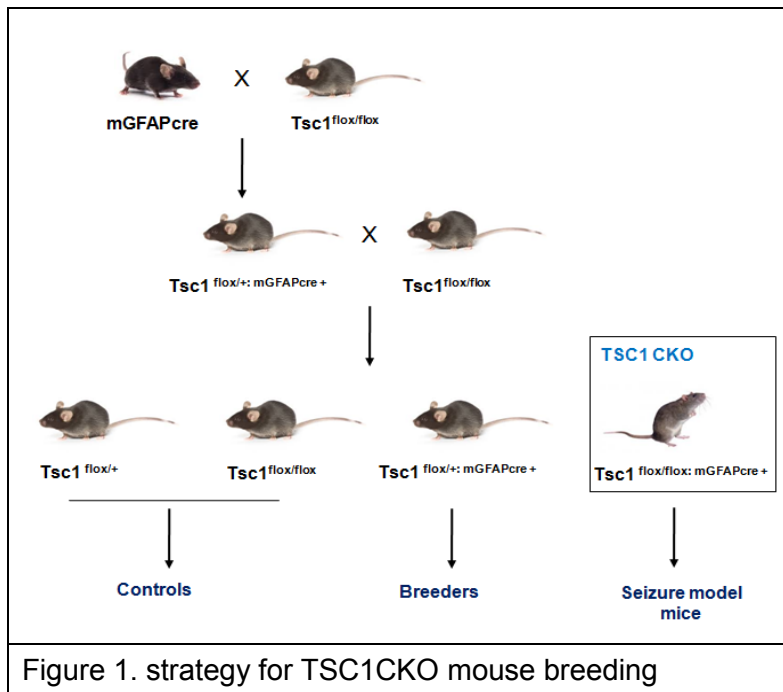
Recent work has directly indicated a role for astrocyte dysfunction in TSC epilepsy. TSC1 conditional knockout mice that specifically targets astrocytes with a human glial fibrillary acidic protein (hGFAP) promoter exhibit spontaneous electrographic and clinical seizures by two months of age. The brains of TSC1 hGFAPCre CKO mice exhibit diffuse histological abnormalities, including glial proliferation and neuronal disorganization with no tubers, indicating that TSC1 deletion in astrocytes is sufficient to cause seizures independent of tubers and cortical dyslamination. The astrocytes from these mice also exhibit altered glial glutamate transport with decreased GLT-1 and GLAST protein expression and impaired potassium buffering with decreased expression of inward rectifier potassium (Kir) channels subunits. Thus, both abnormal astrocytic potassium buffering and impaired glutamate transport are implicated in the neuronal hyperexcitability of TSC conditional knockout mice.

In addition to the glutamate transport and potassium uptake hypothesis, we further propose that abnormal cells in nontuber cortex might form an abnormally excitable network that underlies seizure generation in TSC. Epileptogenesis in non-tuber neural tissue in TSC may arise by an imbalance of decreased inhibitory and increased excitatory synaptic transmission. A deficiency of GABAergic interneurons, downstream from altered mTOR activity in astrocytes, may explain the early onset and severity of seizures in TS: this is consistent with the efficacy of vigabatrin, an inhibitor of GABA transaminase that prevents spasms in 95% of TS infants. Astrocytes could also regulate neuronal excitability by other means that alter expression and function of synaptic receptors for glutamate, or by altering the number of synapses. Defects in neocortical synapse elimination may result in enhanced excitatory synaptic connectivity that underlies enhanced epileptiform activity and seizure susceptibility. We are thus testing these hypotheses using an astrocyte specific TSC deficient mouse model.

## Key Research Accomplishments and Reportable Outcomes

We have combined these two sections, as the key research accomplishments are also reportable outcomes.

### Aim 1. Analyze how cortical astrocyte function is altered in TSC mutant mice.



We hypothesize that enhanced mTOR activity blocks normal function of cortical and hippocampal protoplasmic astrocytes. However, Cre recombinase is expressed in the radial glia of the hippocampal anlage at E12 and in the cortical radial glia at E13.5–14 in the *hGFAP-Cre* mouse. Both populations give rise to neuronal and glial progeny in adult cortex and hippocampus. Using the *hGFAP-Cre* mouse allows removal of TSC genes from these progenitor cells and their neuronal and glial progeny, creating a model with a mixed cell population lacking TSC genes. To address a specific role of astrocytes in regulating synaptic function during development, we used a mouse GFAP (*mGFAP*) promoter sequence directing expression of a Cre-recombinase in most astrocytes of the healthy brain and spinal cord and a subpopulation of the adult stem cells in the subventricular zone. There is no targeting of postnatal or adult neural stem cells or their

progeny in the hippocampus or other brain regions, rendering these mice particularly useful for selective targeting of astrocytes. Figure 1 presents the mouse breeding strategy.

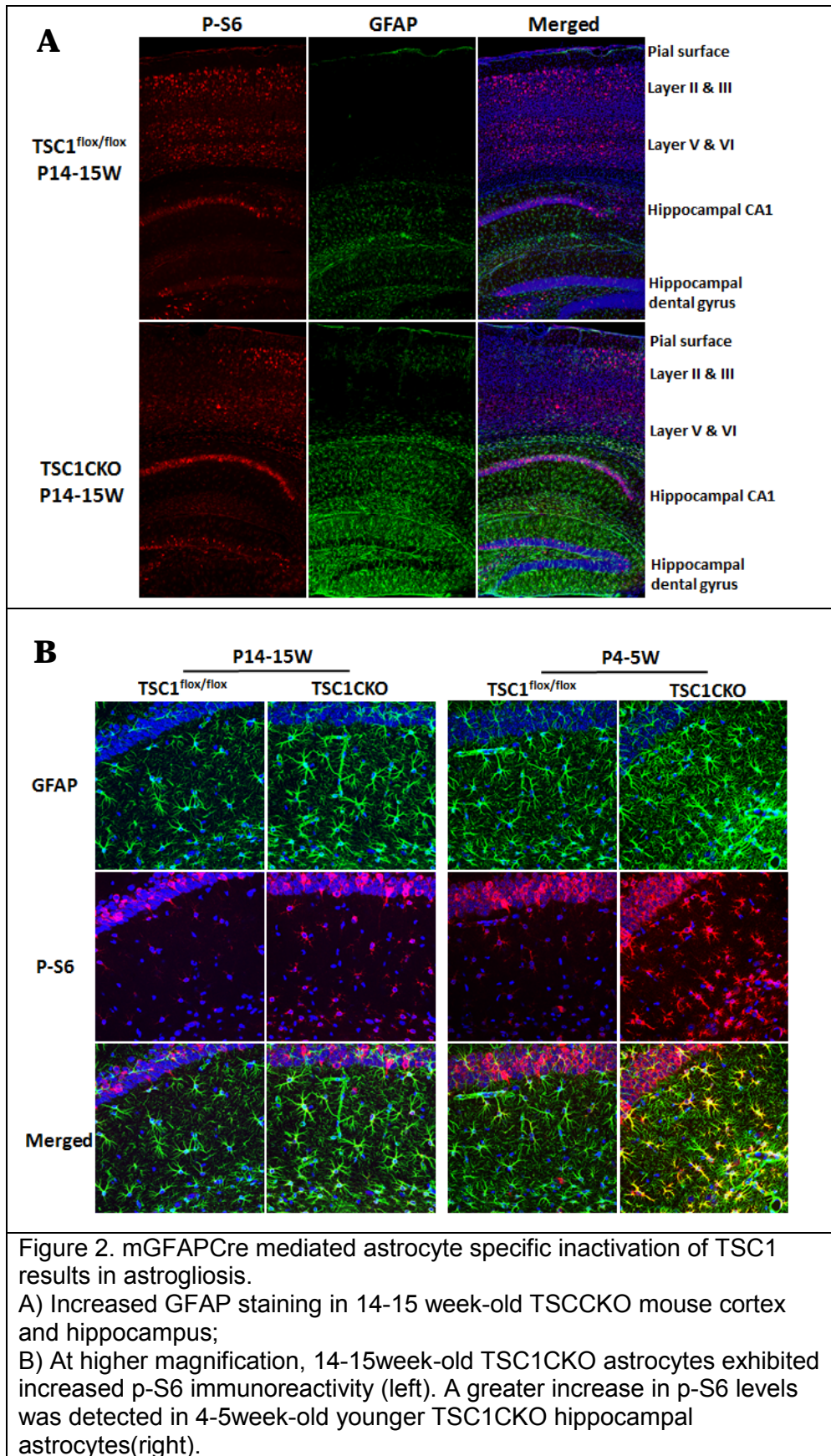
#### 1. TSC1CKO mice develop spontaneous seizures

In adult TSC1CKO mice, we observed frequent jerking of head and body, chewing, and infrequently progression to a tonic-clonic seizure (extension of fore- and hindlimbs, falling on one side, bouncing in the cage). The episodes last for 1-2 mins and are followed by complete physical inactivity. All witnessed seizures occurred in mice upon some kind of stress (changing cages, setting up matings, changing housing room), and the behavior of mice returned to normal after the seizure. Note that females develop seizure 1-2 weeks after weaning (P20-21), while males show first sign of seizure around the age of 2 months.

Our next step is to examine whether spontaneous seizures were accompanied by abnormal brain EEGs and quantify the seizure severity, using video-EEG technique which records EEG and seizure behavior simultaneously in moving animals. We will also assign seizure scores to individual TSC1CKO mouse based on the Racine scale (0 = no behavioral alteration; 1 = immobility, mouth and facial movements, facial clonus; 2= head nodding, forelimb and/or tail extension, rigid posture; 3= forelimb clonus, repetitive movements; 4= rearing, forelimb clonus with rearing, rearing and falling; 5= continuous rearing and falling, jumping; 6= severe tonic-clonic seizures).

#### 2. Increased astrogliosis and mTORc1 signaling in TSC1CKO mice

Tuberlin and hamartin function within a multiprotein complex with both partners required for full mTORC1 inhibition. To assess mTORC1 activity in the cortex, we examined phospho-S6 levels in cortical sections of *Tsc1mGFAP-Cre* CKO and littermate control mice. While a basal level of phospho-S6 expression was seen in neurons from control brains, greatly increased expression was seen throughout the cortex in *Tsc1mGFAP-Cre* CKO mice (Fig. 2B).

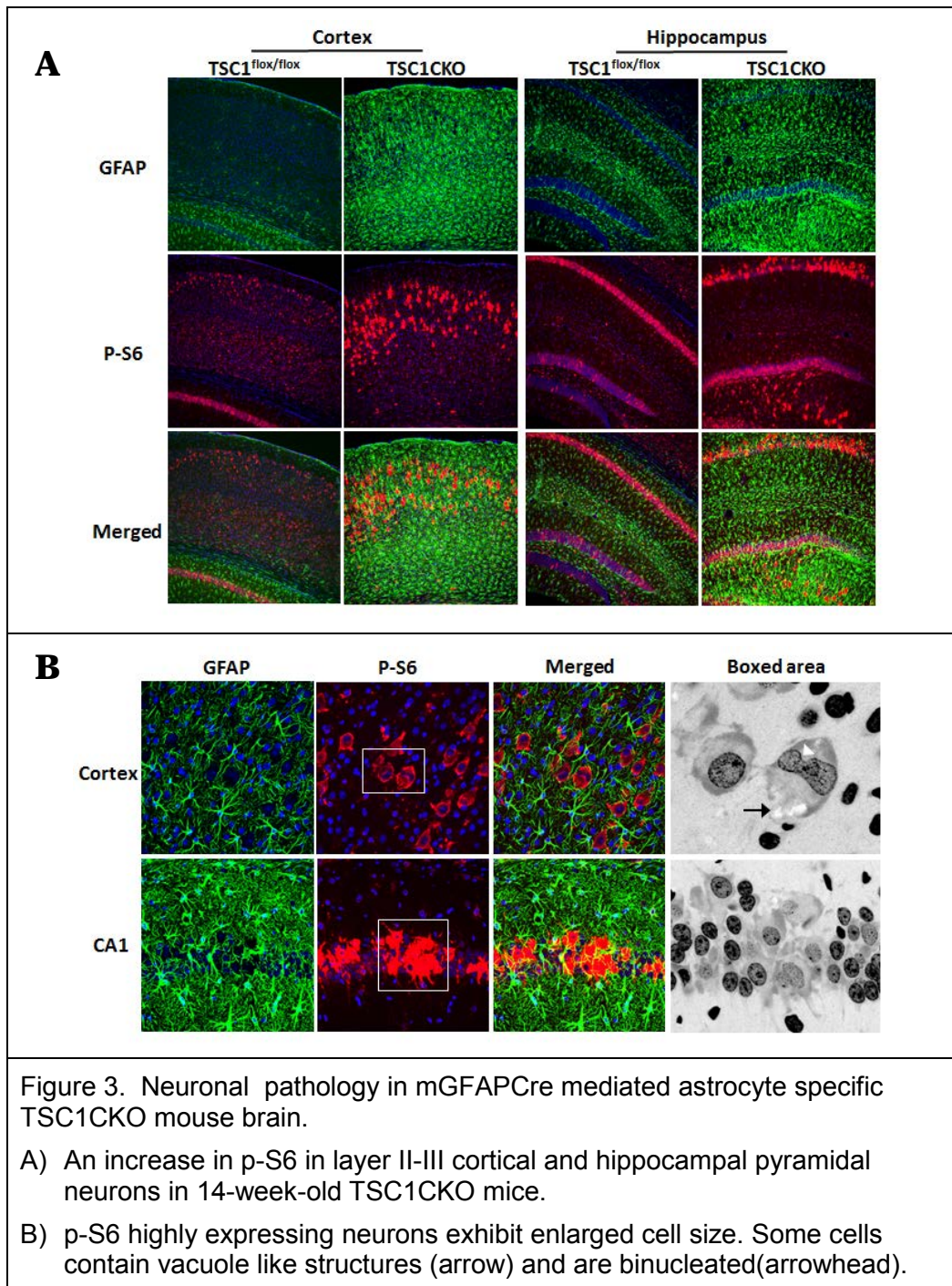


No significant changes in brain size were detected in *Tsc1mGFAP-Cre* CKO mice at the age of 4 weeks, 8 weeks and 14 weeks (not shown). Immunohistochemical staining using a GFAP specific antibody demonstrated an increase in the number of GFAP-immunoreactive cells in the brains from TSC1CKO mice, compared to controls (Figure 1A). Most of these reactive astrocytes express higher level of p-S6 (Figure 1B). However, we did not observe a progressive increase in p-S6 with age in reactive astrocytes. In contrast, we noticed that TSC1CKO astrocytes showed highest p-S6 level at the age of 4 weeks. Though remained higher in TSC1CKO astrocytes, p-S6 level decreases with age in both TSC1CKO and control astrocytes (Figure 2B).

### 3. p-S6 positive Giant neurons in TSC1CKO mouse brain

Phospho-S6 staining revealed a striking dysmorphic appearance and increased cell size in the TSC1CKO cortex (Figs. 3). These enlarged cells were found throughout the layer II/III cortex and hippocampus in some TSC1CKO mice, and in other knockout mice, though quite few, scattered around in different cortical layers. They appeared to be primarily neuronal given co-expression with NeuN and SMI311 (not shown). In addition, we observed occasionally that some giant neurons contained vacuole-like structures and binucleated nuclei (figure 3B), which fit the criteria for cortical tuber dysplastic neurons.



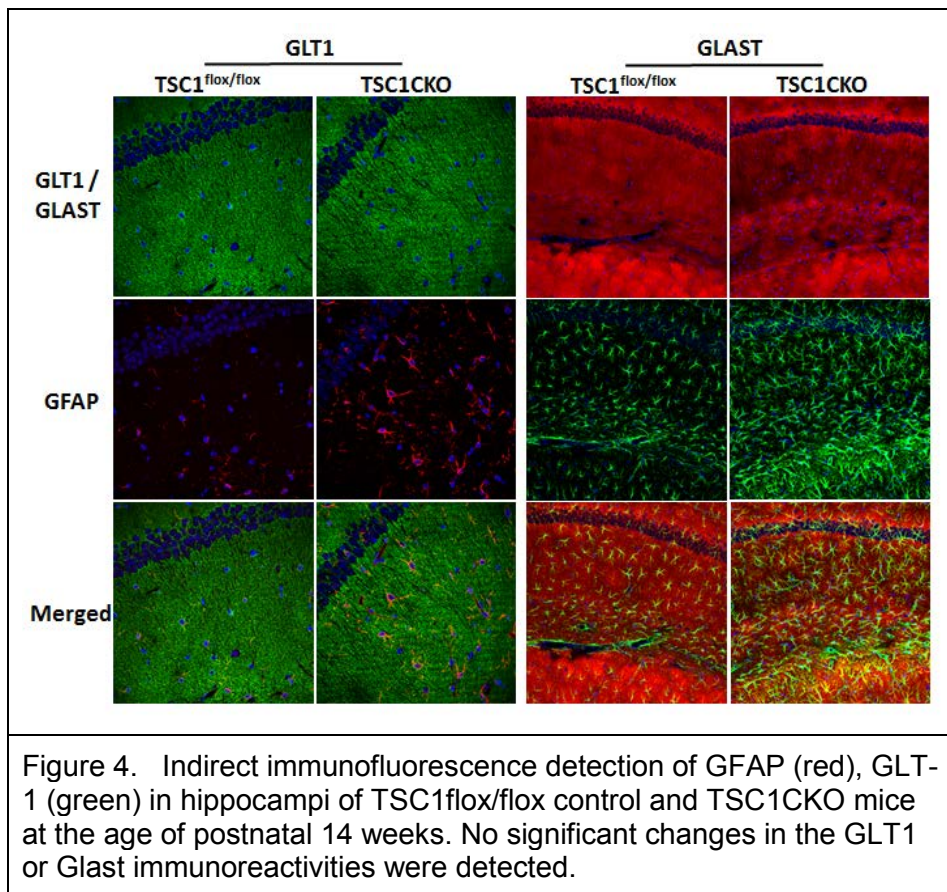




#### 4. Glutamate transport and potassium buffering are intact in TSC1 mGFAPCre CKO astrocytes

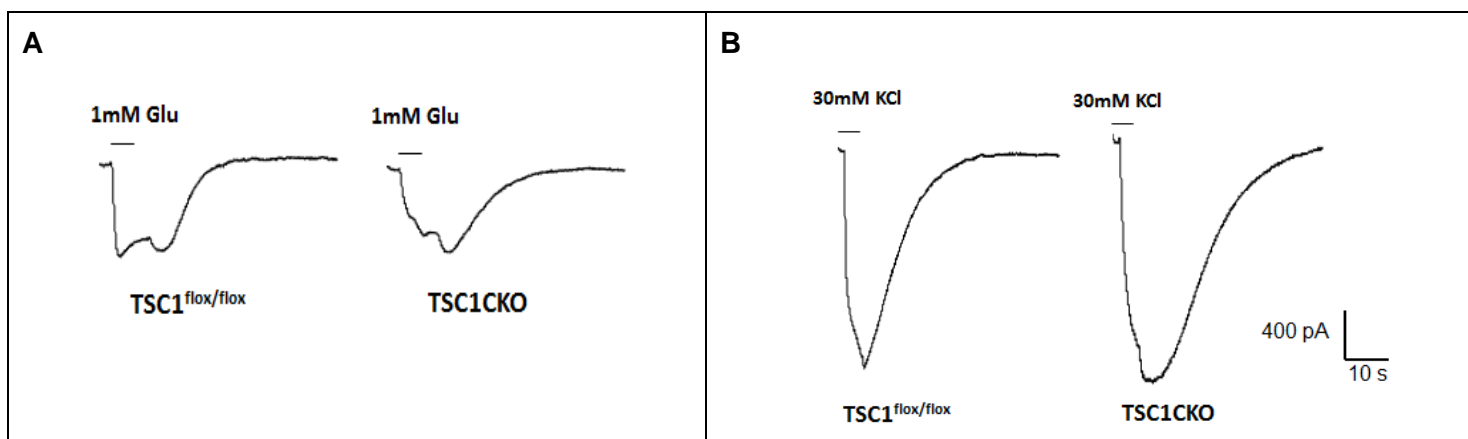
One hypothesis for epilepsy is a failure of astrocytes to maintain the proper extracellular environment required for optimal neuronal function. Astrocytes can take up and buffer extracellular excitatory substances, such as glutamate and potassium. There are two main subtypes of glutamate transporters in astrocytes of the mammalian forebrain and cerebellum: GLAST (EAAT1) and GLT1 (EAAT2). Increased levels of extracellular glutamate have been reported in epilepsy patients, and mice lacking the astrocyte glutamate transporter GLT1 develop seizures. Furthermore, glial specific TSC1 knockout mice, mediated by human GFAP promoter driven cre recombinase, showed deficient astrocyte glutamate transporters and develop seizures. These findings imply that abnormal astrocyte glutamate homeostasis contributes to neurological abnormalities in TSC.

To determine whether TSC1 deficiency in astrocytes in our mouse model also causes glutamate transport deficiency, we first examined hippocampal sections from 14 week-old mice that had undergone spontaneous seizures. Sections double stained with anti-GFAP and GLT-1 (Figure 4, left) or GLAST (Figure 4, right) demonstrated markedly stronger GFAP staining and enlarged astrocytes with thickened processes in the TSC1CKO mice compared with their normal appearance in control mice. No appreciable loss of GLT1 or GLAST immunoreactivity was observed in TSC1CKO mice (Figure 4). We also analyzed the immunoreactivity of GLT1 and GLAST in younger mice (4 week old and 8 week old) absent of spontaneous seizures, and failed to detect the changes in the levels of GLT-1 or GLAST in TSC1CKO mice (data not shown).



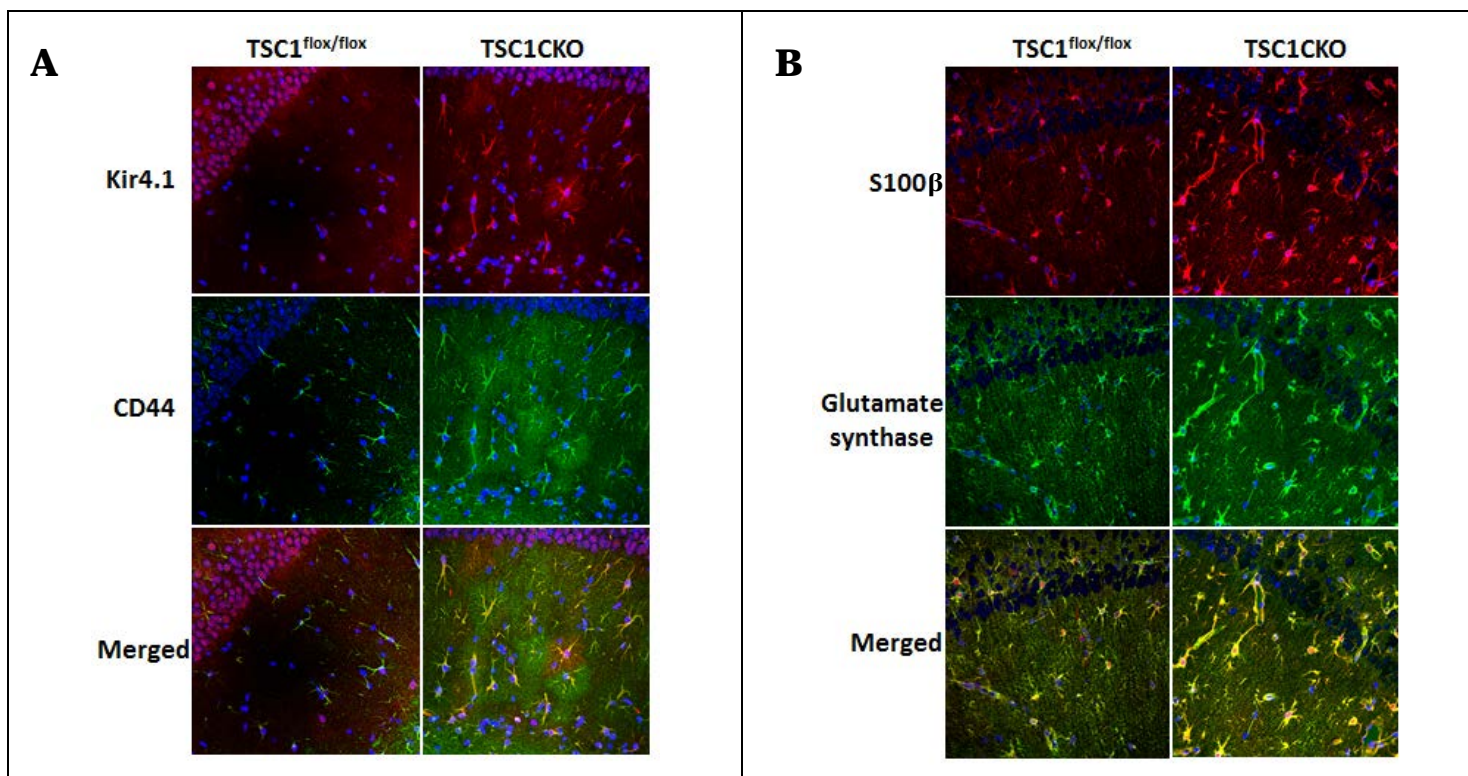
The application of glutamate to astrocytes evokes an inward current caused by activation of astrocyte glutamate transporters and translocation of a net positive charge, including  $\text{Na}^+$  and glutamate. To test whether TSC1 deficient astrocytes display a decrease in glutamate transport function, we measured glutamate induced current under voltage clamp conditions ( $V_m$ ,  $-70$  mV). In these experiments, slices were exposed to a mixture of GluR antagonists (50  $\mu\text{mol/L}$  6-nitro-7-sulfamoylbenzo [f]quinoxaline-2,3-dione, 500  $\mu\text{mol/L}$  (S)- $\alpha$ -methyl-4-carboxy-phenyl-glycine, 40  $\mu\text{mol/L}$  3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid) to block any currents caused by GluR activation. However, we failed to observe significantly reduced glutamate-induced current (Figure 5A), which indicates an intact ability for glutamate uptake in TSC1CKO astrocytes.

In addition to glutamate homeostasis, buffering of extracellular potassium by astrocytes is critical for preventing excessive excitation of neurons. Impaired extracellular potassium uptake by astrocytes can contribute to neuronal hyperexcitability and epileptogenesis. Cultured astrocytes from human GFAP cre driven TSC1CKO mice exhibited reduced Kir currents and decreased expression of specific Kir channel protein subunits, Kir2.1 and Kir6.1. In addition, hippocampal slices from TSC1 human GFAPCre CKO mice exhibited decreased astrocytic Kir currents, as well as increased susceptibility to potassium-induced epileptiform activity.



**Figure 5** Representative recording of glutamate transporter activity (A) and potassium induced current (B) in control and TSC1CKO hippocampal slices. Compared with control astrocytes, TSC1CKO astrocytes show intact glutamate uptake and potassium buffering.

In order to confirm whether the potassium buffering function of astrocytes is deficient in our astrocyte specific TSC1KO mice, we also examined the potassium induced current. In contrast, the ability of TSC1 mGFAPcre CKO astrocytes to take up potassium at high external potassium concentration was not significantly diminished (Figure 5B). Consistent with this finding, we observed a slightly increase in the glial inwardly rectifying potassium channel subunit, Kir4.1, in TSC1CKO hippocampal sections.



**Figure 6** Astrogliosis in TSC1CKO hippocampus. (A) immunohistochemistry of Kir4.1 and CD44 in control and TSC1CKO hippocampal sections; (B) immunohistochemistry of astrocyte makers S100β and glutamine synthetase in TSC1CKO hippocampal sections.

## 5. Increased expression of astrocyte markers CD44, GS and S100 $\beta$

To further characterize the TSC1 deficient astrocytes, we examined the expression of CD44, a hyaluronan receptor expressed in white matter and subpial astrocytes in the normal. In control mice CD44 immunostaining remained within the borders of the stratum lacunosum moleculare (Figure 6A). In contrast, in TSC1CKO mice, CD44 positive astrocytes occupied most of the stratum radiatum. S100 $\beta$ , an astrocyte specific cytosolic proteins, has been considered a marker of the reactive status of astrocytes and was also increased in TSC1CKO astrocytes (Figure 6B).

It has been hypothesized that the loss of glutamine synthetase (GS) activity can lead to increased extracellular glutamate concentrations and epileptic seizures. We therefore hypothesized a decrease GS in TSC1CKO astrocytes. In contrast, we observed an increase in GS immunoreactivity in TSC1CKO hippocampal section (Figure 6B). In fact, several forms of acute brain injury are associated with the increased expression of GS and the decreased expression of GLT-1 and/or GLAST, which eventually leading to the accumulation of excitotoxic extracellular glutamate concentrations. Alternatively, high level of extracellular glutamate may induce a prolonged increase of GS expression, and the increased immunoreactivity of GS in the TSC1CKO hippocampal sections may reflect an increase in the synaptic glutamate level.

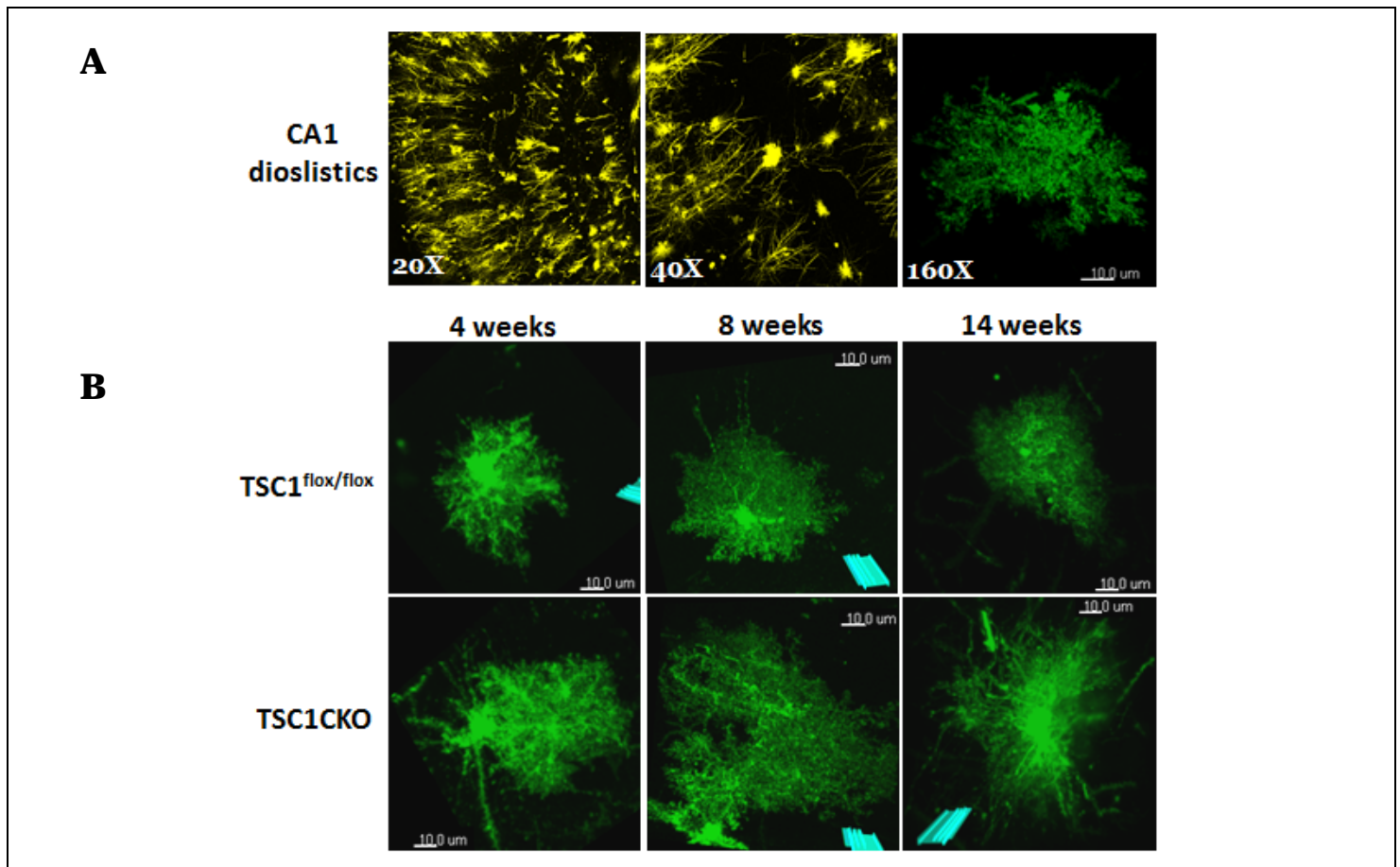


Figure 7. Morphological assessment of TSC1CKO and control astrocytes in the hippocampus. (A) Maximum projections of DiOlistically labeled neurons and astrocytes in CA1 region at 20X (left), 40X (middle) magnification. Single astrocytes can be visualized at 160X magnification (right). Dil labeling revealed fine spongiform processes in individual astrocytes. (B) Developmental changes in astrocyte cell size in control and TSC1CKO mice.

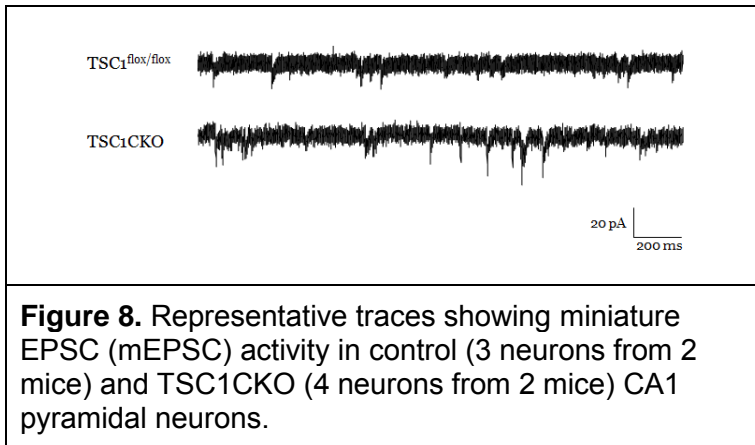


## 6. Increased cell size of TSC1CKO astrocytes

Increased numbers of astrocytes, many with enlarged and dysmorphic shapes, have been observed in TSC mouse models including TSC1 and TSC2 conditional knockouts. Here, we used DiOlistic labeling and 3D reconstruction technique to assess the morphology of TSC1 mGFAPCre CKO astrocytes (Figure 7A). Both control and TSC1CKO astrocytes had a bushy morphology with many fine terminal processes protruding from the main cellular processes (Figure 7B). At 4 weeks of age, both control and TSC1CKO astrocytes show the same cell size. With age, TSC1CKO astrocytes showed an increase in cell size while controls did not. In addition, we found that the number of primary processes leaving the soma was increased in TSC1CKO astrocytes compared with controls. The increased cell size and GFAP intermediate filament network complexity were confirmed in postnatally derived primary astrocyte cultures from TSC1CKO mice (data not shown).

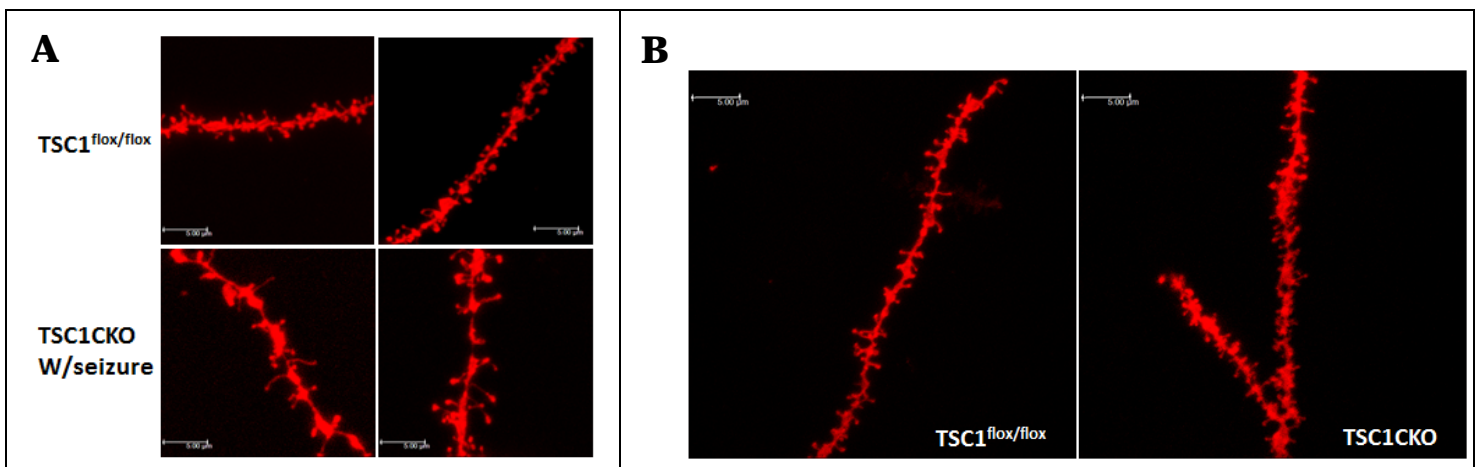
### Aim 2. Analyze whether TSC deficiency in astrocytes causes a postnatal spine pruning defect, resulting in abnormally enhanced excitatory synaptic connectivity and epileptogenicity.

As shown above, astrocyte mediated glutamate homeostasis and potassium buffering remain intact in our TSC1 mGFAPCre CKO mice, which contradicts the glutamate and potassium uptake hypothesis for epileptogenesis in our mouse model. In this section, we tested an alternative hypothesis: astrocyte dysfunction modulates spine density in a non-cell autonomous fashion, which underlies neuronal overexcitation and seizures in TSC.



We first examined the electrophysiological properties of glutamatergic synapses in CA1 pyramidal neurons. Representative traces of miniature EPSCs are shown in Figure 8. These preliminary results imply changes in both amplitude and frequency of miniature EPSC in CA1 pyramidal neurons of TSC1CKO mice, indicative of increased excitatory synaptic transmission.

As a next step, we will further characterize the synaptic transmission of CA3-CA1 synapses, by measuring miniature EPSC, IPSC evoked EPSCs and pair pulse facilitation.



**Figure 9.** Morphological assessment of TSC1CKO and control dendrites of CA1 the hippocampus. (A) Basal dendritic segments (63X, zoom 10X) from 9 week old *TSC1flox/flox* (2 mice) and *TSC1CKO* (2 mice) hippocampal CA1 pyramidal neurons. Compared to controls, less spines but more filapodia were seen in seizure damaged TSC1CKO dendrites. (B) Basal dendritic segments (63X, zoom 6X) from 6 week old *TSC1flox/flox* (2 mice) and *TSC1CKO* (2 mice) hippocampal CA1 pyramidal neurons. Compared to controls, more spines were seen in TSC1CKO dendrites.

We next examined the spine density in the basal dendrites of hippocampal CA1 pyramidal neurons in control and TSC1CKO neurons using DiOlistic labeling with a Helios Genegun system. At the age of 9 weeks, we identified damaged dendrites with irregular dendritic morphology and decreased spine density in mice with spontaneous seizures (Figure 9A). We then analyzed the spine density in 6 week old control and TSC1mGFAP CKO mice before the first sign of seizures occur. Before seizures, TSC1CKO mice exhibit higher spine density in CA1 pyramidal neurons (Figure 9B). As the increase in spine density occurred before the onset of spontaneous seizures, it's likely that an increase in synaptic density underlie the neuronal overexcitability and seizures.

## Conclusions

In this section, we summarize the conclusions shown above and the next steps in our research.

In summary, the major findings in our animal models as detailed above are that a) TSC1 mGFAPCre CKO mice develop spontaneous seizures; b) TSC1CKO mice show astrogliosis, activated mTOR signaling, enlarged cells size for neurons and astrocytes; C) glutamate transport and potassium buffering are intact in TSC1 mGFAPCre CKO astrocytes; D) TSC1CKO mice exhibit abnormal excitatory synaptic transmission; E) the mutants possess synaptic damage induced by spontaneous seizures and F) the mutants display increased spine density before the occurrence of spontaneous seizures.

Following the characterization of mutant astrocytes, we will assess the effect of astrocyte TSC deficiency on spine development in cortical neuronal co-cultures, using the Sulzer lab's established approaches. Cortical neurons will be co-cultured with wild type and TSC2+/- or TSC1-CKO astrocytes. Dendrites and spine morphology will be assayed during maturation *in vitro* at 4, 7, 14 and 21 days in vitro (DIV) by immunostaining for dendritic marker MAP2 and postsynaptic density protein PSD95 and imaged by confocal microscopy. Synaptic density and the number of functional synapses will be represented the frequency of colocalization of the pre- and post-synaptic markers synaptophysin and PSD95. Inhibitory and excitatory synapse formation will be examined using antibodies against synaptophysin to label presynaptic terminals, vesicular glutamate transporter (VGluT) to distinguish excitatory terminals, vesicular GABA transporter (VGAT) for inhibitory terminals and the GABAAR subunits to visualize postsynaptic receptors.

To confirm the effects of TSC1 deficient astrocytes on dendrite and spine morphology *in vivo*, we will label pyramidal neurons in brain slices from astrocyte specific TSC1mGFAP CKO with DiOlistic labeling. We will also immunostain and immunoblot these brain slices for pre- and postsynaptic markers. Dendritic spines in sensory cortex layer V and hippocampal CA1 pyramidal neurons from wild type and TSC1 KO mice will be examined during the primary period of developmental pruning (postnatal day 21, 30 and 60) using DiOlistic labeling with a Helios Genegun system. Spine pruning will be represented by the difference of spine densities between P21 and P30 or P60.

To assess pathophysiological consequences of increased spine density and enhanced excitatory synaptic transmission in TSC1 KO, we will examine seizure susceptibility *in vitro* and *in vivo* (video-EEG). To characterize pre- and post-synaptic functions in hippocampal CA1 pyramidal neurons from wild type and TSC1mGFAP CKO mice, paired pulse facilitation (PPF) of excitatory postsynaptic currents (EPSCs) will be recorded to address the changes in pre-synaptic release probability. To directly compare glutamatergic and GABAergic synaptic transmission, excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) will be recorded by alternately clamping at AMPA or GABAA receptor channel reversal potential.

We will initiate Aim3 "Analyze tuber, peri-tuber and non-tuber tissue from TS and control human brain tissue for mTOR, astrocyte function and synaptic formation" by requesting biopsied and autopsied tuber, peri-tuber and non-tuber brain tissue from TS patients and age-matched control cortex will be obtained from the Maryland Brain Bank. Both fixed and frozen tissue will be subjected to biomarkers for astrocyte function and excitatory and inhibitory synaptic transmission. Neuronal morphology (dendrites and spines) will be analyzed using the modified Golgi method.